ACUTE EFFECTS OF ORGANOPHOSPHOROUS COMPOUNDS ON THE OVINE FETUS

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FINAL REPORT

June 19, 2001

SUPPORTED BY

OFFICE OF NAVAL RESEARCH

GRANT NUMBER N00014-98-1-0730

DOD DISTRIBUTION STATEMENT

APPPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT DATE	3. DATES COVERED *(From - To)*
June 22, 2001	Technical	01 Jun 1998 – 22 Jun 2001

4. TITLE AND SUBTITLE
Acute effects of Organophosphorous compounds on the ovine fetus

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-98-1-0730

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
98PR06350-00

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
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700 W. Olney Road
Norfolk, Va. 23507

10. SPONSOR/MONITOR'S ACRONYM(S)
EVMS

11. SPONSORING/MONITORING AGENCY REPORT NUMBER

12. DISTRIBUTION AVAILABILITY STATEMENT
APPROVED FOR PUBLIC RELEASE; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal is to achieve a better understanding of the toxicity to the fetus of organophosphorous compounds. In ovine plasma, paraoxon inhibition of maternal acetylcholinesterase was 100-fold below that of the fetus. 2-PAM reduced paraoxon-induced inhibition in maternal plasm by approximately fifty percent but had no effect on inhibition in fetal plasma. Hydrolysis of p-nitrophenyl acetate was similar with plasma from human, baboon (maternal and fetal) and maternal sheep but only one half this rate in fetal sheep. Hydrolysis of procaine was most rapid in human plasma, with baboon plasma (maternal and fetal) 4-5 fold lower. Unexpectedly, neither maternal nor fetal plasma hydrolyzed procaine. Hydrolysis of paraoxon in plasma from maternal or fetal baboon was undetectable. In sheep, maternal plasma rapidly hydrolyzed paraoxon whereas fetal plasma lacks the ability to hydrolyze this compound.

15. SUBJECT TERMS
Organophsphorous, paraoxon, fetus, toxicity, esterase

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Manford Castle
U	U	U	SAR	14	19b. TELEPONE NUMBER *(Include area code)* 757 446-5634

ABSTRACT

There is minimal information, and considerable controversy, regarding the acute, sub-acute and chronic effects of organophosphorous anticholinesterases on the fetus. The overall goal of the proposed research is to achieve a better understanding of the consequences to the fetus of exposure to nerve agents such as sarin. These studies will provide the basis for developing a protocol to for deal with situations in which pregnant military and civilian women have been exposed to nerve agents. Although we originally proposed to use sarin as the agent to be investigated, paraoxon was substituted.

In ovine plasma, paraoxon inhibited maternal acetylcholinesterase at a concentration approximately 100-fold below that required to inhibit fetal acetylcholinesterase. Preliminary data suggest that, after inhibition of ovine maternal plasma with 1 X 10^{-6} M paraoxon, the addition of 2-PAM reversed this inhibitory effect by approximately fifty percent. However, in similar studies with ovine fetal plasma, the addition of 2-PAM had no effect on the inhibition of acetylcholinesterase by paraoxon.

Hydrolysis of p-nitrophenyl acetate was similar with plasma from human, baboon (maternal and fetal) and maternal sheep. The notable exception is that plasma from fetal sheep exhibited a rate that was approximately one half that of the other specimens.

Hydrolysis of procaine was most rapid in human plasma. In baboons, maternal and fetal plasma exhibited similar rates and were 4-5 fold less than that of human plasma. An unexpected finding was the lack of either maternal or fetal plasma to hydrolyze procaine.

We were unable to detect any hydrolysis of paraoxon in plasma from either maternal or fetal baboon. An interesting observation was the marked differences between maternal and fetal plasma of sheep to hydrolyze paraoxon. Whereas hydrolysis was very rapid in maternal plasma it was undetectable in fetal plasma. Since paraoxonase is a calcium-dependent enzyme, we test the ability of plasma from maternal and fetal sheep to hydrolyze paraoxon under different calcium conditions. The addition of calcium to fetal plasma did not change metabolism, suggesting that a deficiency of calcium was not responsible for the inability to hydrolyze paraoxon. Conversely, treatment of maternal plasma with EDTA to remove calcium resulted in total in total inhibition of hydrolysis of paraoxon.

These studies demonstrate several important principles. In sheep, maternal plasma rapidly hydrolyzes paraoxon whereas fetal plasma lacks the ability to hydrolyze this compound. Preliminary results suggest 2-PAM reduced paraoxon-induced inhibition in maternal plasm by approximately fifty percent but had no effect on inhibition in fetal plasma.

BACKGROUND

The emphasis and focus of this projected has changed for several reasons. Extensive delays were encountered soon after the award was received. The unwillingness of the US Army to provide sarin necessitated total revision of the goals and objectives. There was further delay and confusion in obtaining permission to revise the protocols and to obtain a non-funded extension. A significant amount of time and effort were devoted to administrative aspects involving these issues, thus markedly reducing the time available to devote to the project. As a result of these delays, the co-investigator (Dr. Barbara Hargrave) was unable to participate in the project due to commitments at her institution and the in vivo studies planned with pregnant sheep could not be completed. We have succeeded in expanding certain aspects of the in vitro work and the results of these studies are contained in this report. As discussed in detail later in the report, a portion of the budget that was committed to the in vivo studies was not used and thus returned.

While the intent of the original proposal was to evaluate the effects of chemical warfare agents such as sarin, the work reported here used paraoxon which has similar but less potent properties.

INTRODUCTION

1. *STATEMENT OF THE PROBLEM.*

The overall goal of the proposed research is to achieve a better understanding of the consequences to the fetus of exposure to nerve agents such as sarin. These studies will provide the basis for developing a protocol to for deal with situations in which pregnant military and civilian women have been exposed to nerve agents. Although we originally proposed to use sarin as the agent to be investigated, paraoxon was substituted for reasons noted above.

The physiological and biochemical consequences of exposure of humans and animals to organophosphorous anticholinesterase compound such as paraoxon and nerve agents (soman, sarin, tabun, etc.) have been well documented (Taylor, 1996). There is also general agreement that the acute toxicity of organophosphorous anticholinesterase compounds is due to inhibition of cholinesterases (acetylcholinesterase and butyrylcholinesterase) while the delayed or long-term toxicity is associated with inhibition of an enzyme that has been termed neuropathy target esterase (NTE). However, there is minimal information, and considerable controversy, regarding the acute, sub-acute and chronic effects of organophosphorous anticholinesterases on the fetus. Studies in rats and

rabbits found no evidence of fetal toxicity with either soman or sarin at doses that produced significant toxicity to the mother (Bates et al., 1990; LaBorde et al., 1996). However, these studies employed gross parameters such as fetal death, malformations, and average body weight of the offspring. Other investigators have presented evidence which suggests that the neurotoxic effects of organophosphorous anticholinesterases are much greater on the mother and that the fetus may be protected from organophosphorous anticholinesterase-induced toxicity (Chanda et al., 1995; Santhoshkumar and Shivanandappa, 1994).

Exposure to organophosphorous anticholinesterases has been associated with toxic effects in the fetus. These include inhibition of protein synthesis in fetal guinea pigs after maternal exposure to parathion (Gupta et al., 1984), presumptive neurotoxicity in human offspring due to exposure of the mother to organophosphorous anticholinesterase insecticides (Romero et al., 1989) and in vitro inhibition of fetal brain acetylcholinesterase by organophosphorous anticholinesterase pesticides (Banerjee et al., 1991). Mehl et al. (1994) demonstrated reductions in brain weight of offspring of guinea pigs exposed to trichlorfon or dichlorvos but not to triortho-cresyl phosphate or soman. While these reports are suggestive of fetal toxicity, no studies have been performed in which more sensitive tests such as inhibition of target enzymes or direct measurement of physiological parameters in the fetus have been used to assess the acute and chronic toxicity of organophosphorous anticholinesterases.

An important aspect of maternal-fetal differences in organophosphorous anticholinesterase toxicity that has not been adequately investigated involves blood esterases. There is evidence that maternal and fetal blood esterases may be qualitatively similar but quantitatively different. Although there are no data available on human fetal cholinesterase activity, studies in neonates suggest that activities are approximately 50% that of adults (Lehmann et al., 1957; Zsigmond and Downs, 1971; Ecobiochon and Stephens, 1973). Similar results have been reported in fetal guinea pigs (Chow and Ecobichon, 1975). However, much of the data in humans involved premature infants and the authors of the studies in guinea pigs noted a significant reduction in cholinesterase activity coincident with birth. Bell and Van Petten (1976; 1977) used the chronically-cannulated ovine fetus to investigate maternal-fetal differences in blood esterases. Carboxylesterase was found to be 8-fold higher in maternal blood than in fetal blood. This contrasted to the activity of acetylcholinesterase that was 2-3 fold higher in fetal blood than in maternal blood. Interestingly, there were no maternal-fetal differences in inhibition of cholinesterase by physostigmine, diisopropylfluorophosphate (DFP) or dichlorovos. While many of these investigations suggest maternal-fetal differences in blood esterases, the potential consequences

of these differences are not known since no physiological or toxicological parameters were measured.

On the other hand, as noted by Mortensen et al. (1998a; 1998b), there is substantial evidence that young animals are more susceptible to the lethal effects of organophosphorous anticholinesterases. These investigators have further demonstrated that age-related and tissue-related differences in sensitivity to organophosphorous anticholinesterases are not due to due to differences in the target enzyme (acetylcholinesterase). Thus, extrinsic factors such non-target binding of cholinesterase inhibitors may complicate interpretation of in vitro parameters such as the IC_{50}. The presence of such extrinsic factors may produce a milieu resulting in differences in enzyme inhibition between the mother and fetus that would produce difference in neurotoxicity.

A potential concern in the present proposal is the appropriateness of the pregnant-sheep model for investigations of the toxicity of organophosphorous anticholinesterases. This model is well established in the field of fetal research. While there will undoubtedly be species differences (as there are even between humans and non-human primates), we believe that the proposed studies will provide valuable insights into the mechanisms of toxic effects of sarin and other organophosphorous anticholinesterases on the fetus. Hematological similarities between human and sheep as well as other similarities in physiology (Lipton and Nathan, 1987; De Waele et al., 1988; Schalm et al., 1975) suggest the sheep model is an appropriate model to investigate the toxicity of sarin on the fetus. Although there are no definitive data, indirect observations suggest that sheep may be more sensitive to organophosphorous anticholinesterases than other species. The accidental exposure of livestock to VX at Dugway Proving Grounds in 1968 (Boffey, 1968a; Boffey, 1968b) suggests that sheep may be more sensitive to nerve agents than is other livestock. This apparent enhanced sensitivity of sheep to organophosphorous anticholinesterases may, in fact, be an advantage. This model may be helpful in identifying problems which may be missed or overlooked with less sensitive models. A more sensitive animal model will also be useful in identifying problems that may arise in individuals who are predisposed to organophosphorous anticholinesterase toxicity due to genetic or environmental factors.

B. *HYPOTHESIS AND RATIONALE*

The central hypothesis of this proposal is that the fetus is less susceptible to the neurotoxicity which normally occurs following exposure to nerve agents such as sarin. We have used in vitro models to investigate

mechanisms that may explain differences in maternal and fetal neurotoxicity following exposure to organophosphorous compounds.

C. TECHNICAL OBJECTIVES.

1. To investigate maternal-fetal differences using plasma obtain from maternal and fetal sheep and from maternal and fetal baboons.

2. To investigate in vitro differences between maternal and fetal target enzymes (acetylcholinesterase, carboxylesterase) in the presence of organophosphorous compounds.

3. To evaluate in the ability of organophosphorous compounds to inhibit maternal and fetal plasma acetylcholinesterases.

4. To determine the capability of maternal and fetal plasma enzymes to metabolize prototype esters such as paraoxon, para-nitrophenyl acetate, and procaine.

D. Military Significance.

Exposure of military and civilian personnel to organophosphorous anticholinesterases may occur inadvertently or intentional as a result of terrorist activities. While much is known about the consequences of such exposure to adults, there is little information concerning the acute and long-term effects on the fetus. The effects on the fetus of countermeasures to attenuate the effects of nerve agent exposure have not been investigated. In the event of such exposure to military or civilian personnel, the military establishment will be the first to be called upon to deal with the consequences.

E. Methods

1. Research Plan

Overview of Experimental Design. For studies in vitro, we obtained fresh maternal and fetal blood specimens from our supplier (Thomas D. Morris, Inc., Reisterstown, MD). The specimens were matched (i.e., fetal blood came from the dam who was the mother of the fetus). The cost of obtaining blood specimens from this supplier is much less than maintaining the animals in our facilities. Likewise, matched plasma specimens were obtained from pregnant baboons housed at our institution.

2. Procedures

a) *Biochemical Monitoring*.

1) *Plasma carboxylesterase.* Carboxylesterase activity was measured in maternal and fetal plasma by the method used previously in this laboratory (Castle, 1989; Castle, 1990). This method consists HPLC/UV quantitation of procaine and the hydrolyzed product (p-aminobenzoic acid) produced by carboxylesterase.

2) Plasma Paraoxonase. Paraoxonase activity was determined by HPLC/UV quantitation of paraoxon and the hydrolyzed product (p-nitrophenol).

3) P-Nitrolphenyl Acetate Hydrolysis. Hydrolytic activity was determined by HPLC/UV quantitation of paraoxon and the hydrolyzed product (p-nitrophenol).

4) Plasma Acetylcholinesterase. Plasma cholinesterase was assayed using the colorimetric procedure based upon the Ellman reaction (Dietz et al., 1973).

3. Protocols.

a) In Vitro Studies. Blood, collected from maternal and fetal sheep or baboons, was fractionated into several components (plasma, lymphocytes, erythrocytes and platelets). Plasma was used to investigate cholinesterase and carboxylesterase activity. Substrate was added at concentrations between 10^{-10} M and 10^{-4} M. An aliquot (100 μL) of plasma was mixed with 200 μL of Tris buffer (pH 7.4). Paraoxon in Tris buffer was added to initiate the reaction. The preparation was incubated at 37° C. At specified time intervals (1, 2, 4, 8,16 and 32 minutes), an aliquot of the incubation mixture (40 μL) was removed and immediately mixed with 160 μL of ethanol to stop the reaction and precipitate proteins. After centrifugation, a portion of the supernatant was removed and mixed with HPLC mobile phase. An aliquot of this preparation was injected on the HPLC.

b) HPLC Conditions. All assays employed a Beckman Ultrasphere C_{18} column (25 cm with 10 micron packing material). The mobile phase for paraoxon and p-nitrophenyl acetate assays consisted of methanol:acetonitrile:aqueous buffer (37:37:26), pH 4 with a flow rate of 1 mL/min. Ultraviolet detection was at 274 nm. The mobile phase procaine assays consisted of acetonitrile:aqueous buffer (18:82), pH 4 with a flow rate of 1 mL/min. Ultraviolet detection was at 254 nm.

F. RESULTS

As indicated in Table 1, paraoxon inhibited maternal acetylcholinesterase at a concentration approximately 100-fold below that required to inhibit fetal acetylcholinesterase. If this were the only factor to consider, the implication is that the fetus may be protected from anticholinesterase compounds. Obviously, a number of other factors must be considered such as the ability of drug to reach and accumulate in fetal plasma. Nevertheless, this is one factor that must be considered in evaluating the difference in organophosphorous toxicity between the mother and the fetus.

TABLE 1

INHIBITION OF PLASMA ACETYLCHOLINESTERASE BY PARAOXON

	PERCENT INHIBITION		
PARAOXON CONCENTRATION	Maternal sheep	Fetal sheep	Human
1×10^{-9} M	0	0	0
5×10^{-9} M	0	0	0
1×10^{-8} M	0	0	0
5×10^{-8} M	2	0	0
1×10^{-7} M	18	0	0
5×10^{-7} M	43	0	42
1×10^{-6} M	74	0	76
5×10^{-6} M	86	17	84
1×10^{-5} M	96	68	96
5×10^{-5} M	98	70	99
1×10^{-4} M	96	76	97
5×10^{-4} M	99	91	100
1×10^{-3} M	97	100	100
5×10^{-3} M	99	100	100

Data are expressed as percent inhibition compared to paired control

Although time constraints prevented us from completing studies involving the ability of 2-PAM to attenuate paraoxon-induced inhibition of acetylcholinesterase, we were able to obtain some preliminary data. After inhibition of ovine maternal plasma with 1 X 10^{-6} M paraoxon, the addition of 2-PAM reversed this inhibitory effect by fifty percent. However, in similar studies with ovine fetal plasma, the addition of 2-PAM had no effect on the inhibition of acetylcholinesterase by paraoxon.

The data in Table 2 and Table 3 illustrate the relative ability of plasma from humans, sheep and baboons to hydrolyze p-nitrophenyl acetate. Hydrolysis was similar with plasma from human, baboon (maternal and fetal) and maternal sheep. The notable exception is that plasma from fetal sheep exhibited a rate that was approximately one half that of the other specimens.

TABLE 2

HYDROLYSIS OF P-NITROPHENYL ACETATE (1 X10^{-5} M) BY PLASMA OF DIFFERENT SPECIES

TIME (min)	MATERNAL BABOON	FETAL BABOON	MATERNAL SHEEP	FETAL SHEEP	HUMAN
1	68.34	59.76	89.23	23.26	50.00
2.5	89.45	91.33	95.38	44.19	71.81
5	97.26	100	100	58.62	94.18
10	100	100	100	76.74	98.72

Data are expressed as percent of substrate hydrolyzed.

TABLE 3

HYDROLYSIS OF P-NITROPHENYL ACETATE BY (1 X10^{-4} M) PLASMA OF DIFFERENT SPECIES

TIME	MATERNAL BABOON	FETAL BABOON	MATERNAL SHEEP	FETAL SHEEP	HUMAN
1	46.67	52.32	15.11	3.41	33.35
2.5	73.60	73.47	22.50	6.67	52.38
5	85.66	91.02	90.23	11.82	85.46
10	98.42	97.96	100	47.42	97.35

Data are expressed as percent of substrate hydrolyzed.

The data in Table 4 illustrate the relative ability of plasma from humans, sheep and baboons to hydrolyze procaine. Hydrolysis was most rapid in human plasma. In the baboon, maternal and fetal plasma exhibited similar rates and were 4-5 fold less than that of human plasma. An unexpected finding in sheep was the lack of either maternal or fetal plasma to hydrolyze procaine. Similar results were obtained at other substrate concentrations (1 X10^{-4} M and 1 X10^{-6} M).

TABLE 4

HYDROLYSIS OF PROCAINE (1 $X10^{-5}$ M) BY PLASMA OF DIFFERENT SPECIES

TIME	MATERNAL BABOON	FETAL BABOON	MATERNAL SHEEP	FETAL SHEEP	HUMAN
1	4.38	2.55	0	0	18.56
2.5	10.89	9.41	0	0	44.93
5	19.18	21.42	0	0	90.82
10	42.27	35.74	0	0	100
20	65.36	53.54	0	0	100

Data are expressed as percent of substrate hydrolyzed.

The data in Tables 5-7 illustrate the relative ability of plasma from sheep and baboons to hydrolyze paraoxon. We were unable to detect any hydrolysis of paraoxon in plasma from either maternal or fetal baboon. An interesting observation was the marked differences between maternal and fetal plasma of sheep to hydrolyze paraoxon. Whereas hydrolysis was very rapid in maternal plasma but was undetectable in fetal plasma.

TABLE 5

HYDROLYSIS OF PARAOXON (1 $X10^{-6}$ M) BY PLASMA OF DIFFERENT SPECIES

TIME	MATERNAL BABOON	FETAL BABOON	MATERNAL SHEEP	FETAL SHEEP
1	0	0	49.11	0
2.5	0	0	98.12	0
5	0	0	100	0
10	0	0	100	0

Data are expressed as percent of substrate hydrolyzed.

TABLE 6

HYDROLYSIS OF PARAOXON (1 $X10^{-5}$ M) BY PLASMA OF DIFFERENT SPECIES

TIME	MATERNAL BABOON	FETAL BABOON	MATERNAL SHEEP	FETAL SHEEP
1	0	0	12.50	0
2.5	0	0	45.80	0
5	0	0	100	0
10	0	0	100	0

Data are expressed as percent of substrate hydrolyzed.

TABLE 7

HYDROLYSIS OF PARAOXON (1 $X10^{-4}$ M) BY PLASMA OF DIFFERENT SPECIES

TIME	MATERNAL BABOON	FETAL BABOON	MATERNAL SHEEP	FETAL SHEEP
1	0	0	13.33	0
2.5	0	0	23.35	0
5	0	0	49.78	0
10	0	0	84.33	0

Data are expressed as percent of substrate hydrolyzed.

Since paraoxonase is a calcium-dependent enzyme, we test the ability of plasma from maternal and fetal sheep to hydrolyze paraoxon under different calcium conditions. The addition of calcium to fetal plasma did not change metabolism, suggesting that a deficiency of calcium was not responsible for the inability to hydrolyze paraoxon. Conversely, treatment of maternal plasma with EDTA to remove calcium resulted in total in total inhibition of hydrolysis of paraoxon.

G. DISCUSSION

In sheep, there appear to be two important differences between the mother and the fetus in response to organophosphorous-induced inhibition of acetylcholinesterase.

Firstly, maternal plasma rapidly hydrolyzes paraoxon whereas fetal plasma lacks the ability to hydrolyze this compound. On one hand, the ability of maternal plasma to hydrolyze paraoxon may lead to protection of the fetus by rapidly reducing maternal blood levels and thus the concentration gradient from the mother to the fetus. On the other hand, the inability of the fetus to metabolize paraoxon could lead to enhanced fetal toxicity due to markedly prolonged half-life of the drug that does reach the fetal circulation.

Secondly, there appears to be maternal-fetal differences in the ability of reactivators such as 2-PAM to reverse the inhibition of acetylcholinesterase caused by organophosphorous compounds. Preliminary results, suggest 2-PAM reduced paraoxon-induced inhibition in maternal plasm by approximately fifty percent but had no effect on inhibition in fetal plasma. This may have important implications in situations in which the fetus has been exposed to organophosphorous compounds. Attenuation of toxicity in the mother following administration of antidotes such as 2-PAM may be misleading if one assumes that similar effects are being produced in the fetus.

The current studies provide further evidence that we must be cognizant of species differences in assessing the effects of drugs.

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